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Chemical Activation of Non-Mutagenic Nitrated Polycyclic Aromatic Hydrocarbons to Mutagens

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16. ABSTRACT

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The chemical activation of non-mutagenic nitrated polycyclic aromatic hydrocarbons to mutagens

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Summary

Nitrated polycyclic aromatic hydrocarbons, including carcinogens, may be non-mutagenic in microorganisms because bacterial nitroreductases are unable to reduce their nitro function to proximate mutagenic hydroxylamines. This reduction of the nitro moiety can be accomplished chemically in situ using zinc dust. The procedure, which is compatible with the Salmonella mutagenicity assay, was used to generate mutagens from chemicals which otherwise are non-mutagenic even in the presence of microsomal preparations.

Nitro-containing chemicals are receiving increased attention as a result of the detection of nitrated polycyclic aromatic hydrocarbons (PAHs) in the environment mainly as a result of the facile acid-catalyzed nitration of PAHs which arise from incomplete stationary and mobile combustion processes (for references, see Mermelstein et al., 1981). Because some of the nitrated PAHs are among the most potent mutagenic substances reported in the literature (Mermelstein et al., 1981; Rosenkranz et al., 1980b; Weill-Thevenet et al., 1981), these findings have resulted in: (a) a concern regarding the possible health risks of exposure to this ubiquitous group of substances; and (b) a renewed interest in the mechanism of action of nitro-containing chemicals.

Abbreviations: 1,8-DND, 1,8-dinitronaphthalene; 5-NQ, 5-nitroquinoline.

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Studies on the mutagenicity in bacteria of nitro PAHs have shown that microorganisms possess uniquely potent nitroreductase enzymes which presumably activate these chemicals to penultimate hydroxylamine mutagens (Mermelstein et al., 1981; Rosenkranz et al., 1981, Rosenkranz and Mermelstein, 1980). Eukaryotes, on the other hand, either lack these enzymes or have only low levels of them. The further discovery that bacteria possess a family of nitroreductases with varying specificities for nitro PAHs (Mermelstein et al., 1981; McCoy et al., 1981a,b; Rosenkranz et al., 1981) suggested that prokaryotes have much broader capability to activate nitro compounds to mutagens. This is turn raised questions concerning the relevance of microbial assays in assessing the health hazards of such chemicals, although it must be remembered that ecologically the relationship between man and bacteria is an intimate one and cooperative metabolic processes between bacterial and mammalian enzymes in the generation of ultimate mutagens have been described (Rosenkranz et al., 1980a; Karpinsky and Rosenkranz, 1980; Karpinsky et al., 1979; McCoy et al., 1979).

On the other hand, however, a number of nitro PAHs and nitrated polyheterocyclics have been described which are not mutagenic for bacteria (Chiu et al., 1978; McCoy et al., 1981c; Mermelstein et al., 1981) and yet these compounds may be carcinogenic. These observations, therefore, raise the possibility that there are some nitro-containing chemicals which, although carcinogenic, are not detected as mutagens in bacteria, which of course has implications in the application of short-term tests for predicting carcinogenicity. The present study was undertaken to determine the basis of the non-mutagenicity of such chemicals, and more specifically whether: (a) the bacteria were unable to activate the chemical to proximate mutagens (presumably the hydroxylamino analog); or (b) whether the putative intermediate that is formed is not mutagenic. A series of nitroquinolines and 1,8-dinitronaphthalene were chosen as model chemicals. This choice was dictated by the earlier findings that while 5- as well as 6-nitroquinoline are direct-acting bacterial mutagens (Chiu et al., 1978; Epler et al., 1977; Nagao et al., 1977; Mermelstein et al, 1981), the carcinogenic 8-nitroquinoline (8-NQ) (Takahashi et al., 1978) is not. 1,8-Dinitronaphthalene was included in the study because it can be considered an analog of 8-nitroquinoline and unlike other nitrated naphthalenes it, too, is not a mutagen in bacteria (McCoy et al., 1981c; Mermelstein et al., 1981).

Materials and methods

Chemicals. The nitroquinolines and nitronaphthalenes were obtained from Aldrich Chemical Co. zinc dust was from Merck and Co., and ammonium chloride from Fisher Scientific Co.

Microbial mutagenicity assay. The Salmonella mutagenicity assay was carried out as described by Ames et al. (1975). When the "pre-incubation" procedure was used, 0.1 ml of bacteria were incubated with the test chemicals at 37°C for 0.75 or 1 h whereupon 2 ml of top agar was added, the suspension mixed and poured onto agar plates for enumeration of revertants following 2 days of incubation at 37°C.

Every dilution was tested in triplicate on at least 2 separate occasions. Overnight cultures were used.

The properties of the nitroreductase-deficient strains TA100NR and TA98NR have been described earlier (Rosenkranz et al., 1981; Rosenkranz and Poirier, 1979; Rosenkranz and Speck, 1975; Speck et al., 1981). They retain plasmid pKM101 and the deep-rough character, and they are deficient in a nitroreductase which reduces nitrofurans, niridazole, nitronaphthalenes and nitrofluorenes to mutagens (Rosenkranz and Mermelstein, 1980).

Chemical reduction. For the reduction using zinc dust, the chemical was dissolved in dimethylsulfoxide while NH_4Cl and zinc dust were made up in aqueous media (ratio of DMSO to H_2O, 1:2.5). When anaerobic conditions were required, the reaction mixtures were placed into Gaspak Jars (BBL, Cockeysville, MD).

Results and discussion

In confirmation of earlier reports (Chiu et al., 1978; Epler et al., 1977; Nagao et al., 1977), 8-NQ was found to be devoid of direct-acting mutagenicity for *Salmonella typhimurium* while both 5-nitroquinoline (5-NQ) and 6-nitroquinoline (6-NQ) exhibit direct-acting activity primarily for strain TA100 and to a much lesser extent for strain TA98, the indicator strain for frame-shift mutations (Table 1). This is in further concordance with the previous findings that bicyclic chemicals capable of forming DNA adducts (e.g., naphthalene derivatives, 4-nitroquinoline 1-oxide) induced mutation of the base-substitution type while tricyclic chemicals capable of forming such covalent derivatives induced primarily mutations of the frame-shift variety (e.g., fluorene, anthracene and acridine derivatives) (Rosenkranz and Mermelstein, 1980; Mermelstein et al., 1981).

It is notewhorthy, however, that while the base-substitution activity of 6-NQ was detected both in strain TA1535 and its plasmid-containing derivative TA100, the mutagenicity of 5-NQ was evident only in strain TA100. In this respect, therefore, the mutagenicity of 5-NQ is reminiscent of that of nitrofurans which is evident only in plasmid-containing strain (i.e. TA100). However, perhaps even more significant is the fact that a similar finding was made with 1- and 2-nitronaphthalene (Table 2). Thus 2-nitronaphthalene, which might be thought of as an analog of 6-NQ, was mutagenic for both TA100 and TA1535 while 1-nitronaphthalene and 1,5-dinitronaphthalene, the congeners of 5-NQ, required the presence of the plasmid to express their activities (i.e., active only in TA100) (McCoy et al., 1981c; Mermelstein et al., 1981). Because plasmid pKM101, the element present in TA100, codes for an error-prone DNA repair enzyme, it would appear that the chemical change in DNA induced by 5-NQ is recognized by such an enzyme. In this connection, it should be noted that the activity of 5-NQ for TA100 exceeds that of 6-NQ in the same tester strain which is in accord with this possibility.

The mutagenicity of both 6-NQ and 5-NQ for *Salmonella typhimurium* appears to depend upon the enzymic reduction of the nitro function to the corresponding hydroxylamine as tester strains deficient in the "classical" nitroreductase, the

TABLE I

MUTAGENICITY OF NITROQUINOLINES

Chemical	Amount (μg per plate)	Revertants per plate				
		TA1535	TA100	TA100NR	TA98	TA98NR
5-Nitroquinoline	0	12	165	140	10	10
	0.3	15	122		11	
	1.0	14	121		16	
	3.3	16	145		15	
	10.0	20	145	146	20	15
	33.3	19	176	155	33	16
	100	24	298	150	75	19
	333	24	653	152	197	14
Rev./μmole		*10*	*197*	*0*	*113*	*0*
6-Nitroquinoline	0.3	14	137		14	
	1.0	16	144		17	
	3.3	16	139		18	
	10.0	25	157	136	23	13
	33.3	48	148	132	30	13
	100	118	195	117	62	15
	333	174	342	109	110	13
Rev./μmole		*184*	*52*	*0*	*90*	*0*
8-Nitroquinoline	10	14	130		15	
	33	15	115		14	
	100	13	140		16	
	333	1	12		13	
Rev./μmole		*0*	*0*	*0*	*0*	*0*

enzyme which recognize the nitro functions of nitronaphthalenes, nitrofluorenes, nitrofurans and niridazole, are not mutagenized by these nitrated quinolines (Table 1).

8-NQ is not directly mutagenic for Salmonella (Table 1), yet following the addition of a post-mitochondrial fraction (S9), mutagenicity, primarily for TA100, is demonstrable (Nagao et al., 1977). Thus it may be that 8-NQ is either not reducible by the bacterial enzyme to 8-hydroxylaminoquinoline, or that because of steric factors the latter (or its electrophilic product) is not capable of forming a covalent DNA adduct. The demonstration of the mutagenicity of 8-NQ in the presence of S9 does not necessarily indicate that such preparations reduce 8-NQ to the corresponding hydroxylamine. Some conceivable metabolites of 8-NQ have been reported to be mutagenic, e.g. 8-hydroxyquinoline, quinoline, 8-hydroxyquinoline 1-oxide, 8-aminoquinoline (Nagao et al., 1977; Epler et al., 1977; Hollstein et al., 1978), others (such as ring oxides) might be mutagenic as well.

Because 8-NQ has been reported to be carcinogenic in rat following oral administration and because tumor distribution was rather specific, indicating probable localized sites of biotransformation (Takahashi et al., 1978), we investigated the possibility that the lack of mutagenicity of 8-NQ in Salmonella was the result of an

TABLE 2

MUTAGENIC ACTIVITIES OF NITRO-CONTAINING CHEMICALS AND THEIR REDUCTION PRODUCTS

Chemicals	Revertants per nmole		
	TA1535	TA100	TA98
1-Nitronaphthalene	0	0.96	0.05
2-Nitronaphthalene	1.4	1.3	0.20
1,8-Dinitronaphthalene	0	0	0
1,8-DNN + Zn dust (1,8-dihydroxylaminonaphthalene)		1.12[a]	
1,5-Dinitronaphthalene	0	4.7	3.30
5-NQ	0.01	0.20	0.11
6-NQ	0.18	0.09	0.05
8-NQ	0	0	0
8-NQ + Zn dust (8-hydroxylaminoquinoline)		2.7[b]	0.39[b]
2-Nitrofluorene	0.01	6.1	13.9
4-Nitroquinoline 1-oxide	120	1399	126
Nitrofurantoin	0	201	

Abbreviations: NQ, nitroquinoline; DNN, dinitronaphthalene.

[a] Calculated from data of Table 4.

[b] Calculated from data of Table 3.

The data on nitronaphthalenes, nitrofurantoin, 2-nitrofluorene and 4-nitroquinoline 1-oxide are taken from McCoy et al., 1981b, 1981c; Mermelstein et al., 1981; and Rosenkranz and Mermelstein, 1980.

inability by bacterial nitroreductases to convert it to the proximate mutagen, the corresponding hydroxylamine. The possibility was explored, therefore, of chemically reducing 8-NQ to 8-hydroxylaminoquinoline. Because of the known oxygen lability of such hydroxylamines which results in reoxidation to the parent chemical, it was thought of advantage, initially, to perform such chemical reductions in situ, i.e. in the presence of the bacteria. This, in turn, necessitated a chemical procedure which is sufficiently mild to permit bacterial survival. Reduction with zinc dust (in the presence of ammonium chloride) (Kamm, 1964; Hickinbottom, 1950) proved to be compatible with the bacterial assay. However, incorporation of the reducing mixture into the agar overlay together with the bacteria and the chemical (Table 3, Expt. I) did not result in the generation of a mutagenic species. On the other hand, pretreatment of a similar amount of 8-NQ (10 μg) in solution with the zinc dust in the presence of the bacteria (45 min, 37°C) yielded a mutagenic product (Table 3, Expt. II). The extent of the mutagenic signal was dependent upon the initial 8-NQ concentration (compare Expts. II and III, Table 3).

In view of the known oxygen lability of the postulated hydroxylamines, attempts were made to generate them under anaerobic conditions and indeed the products formed anaerobically exhibited an enhanced mutagenicity (Table 3, Expts. IV and VI). Additional studies indicated that a 8-NQ/Zn ratio of 1/5 (wt./wt.) appeared optimal for the formation of the mutagenic hydroxylamine (Table 3, Expt. IV).

As expected from earlier studies, the reduced chemical species was primarily of

TABLE 3

MUTAGENICITY OF CHEMICALLY REDUCED 8-NITROQUINOLINE

Expt.	Procedure	Additions	Amount/ plate (μg)	Revertants per plate			
				TA98 (O_2)	TA98 ($-O_2$)	TA100 (O_2)	TA100 ($-O_2$)
I	Plate incorporation	None	0	7		65	
		Zn dust	10	10		78	
		8-NQ	10	14		41	
		8-NQ+Zn	10/10	10		78	
II	Pre-incubation	None	0	9		98	
		Zn dust	10	9		103	
		8-NQ	10	7		117	
		8-NQ+Zn	10/10	25		197	
III	Pre-incubation	None	0	6		64	
		Zn dust	40	10		53	
		8-NQ	40	9		68	
		8-NQ+Zn	40/40	33		207	
IV	Pre-reacted	None	0	9	8	59	72
		Zn	80	8	8		87
		8-NQ	40	11	10	63	93
			80	11	14	47	99
		8-NQ+Zn	20	67	87	134	397
			40	90	132	Toxic	Toxic
			80	197	Toxic	Toxic	Toxic
V	Pre-reacted	None				212	212
		8-NQ	32			186	206
		8-NQ+Zn	8				301
			16				462
			32				584
			64				618
VI	Pre-reacted	None	0			67	89
		Zn-dust	80			88	
		8-NQ	8			76	71
		8-NQ+Zn	4				151
			8			130	242

Abbreviation: 8-NQ, 8-nitroquinoline.

Expt. I: The stock solutions contained either 200 μg 8-NQ per ml, or 200, 200 and 50 μg 8-NQ, Zn dust and NH_4Cl per ml, respectively. 50-μl portions were incorporated into the agar overlay.

Expt. II: 50-μl portions of the stock solutions used for Expt. I were incubated for 45 min at 37°C with 0.1-ml amounts of overnight cultures whereupon soft agar was added and the samples processed.

Expt. III: The stock solutions contained either 800 μg 8-NQ per ml or 800, 800 and 200 μg 8-NQ, Zn dust and NH_4Cl per ml, respectively. 50-μl amounts of these were incubated with 0.1-ml quantities of overnight cultures for 45 min at 37°C.

Expt. IV: Solutions of 8-NQ varying in concentrations from 320 to 1600 μg per ml were incubated with or without Zn dust (1600 μg per ml) and NH_4Cl (400 μg per ml) at 37°C for 24 h either aerobically (O_2) or anaerobically ($-O_2$), whereupon the samples were used either at once or stored at −20°C until assayed.

Expt. V: The stock solution contained 320 μg 8-NQ per ml in the presence or absence of Zn dust (1600 μg/ml) and NH_4Cl (400 μg per ml). Incubation was for 24 h at 37°C.

Expt. VI: The stock solution contained 8-NQ (160 μg per ml) in the presence or absence of the zinc dust (800 μg/ml) and NH_4Cl (200 μg/ml). Incubation was for 24 h at 37°C.

the base-substitution variety, i.e. it preferentially mutagenized strain TA100 (approx. 2.7 versus 0.39 revertants per nmole for strains TA100 and TA98, respectively). The nitroreductase-deficient strains TA100NR and TA98NR, which are not mutagenized by nitroquinolines and nitronaphthalenes were fully responsive to the mutagenic action of the putative 8-hydroxylaminoquinoline (results not shown) indicating that the nitroreductase deficiency was overcome, as would be expected for hydroxylamines (McCoy et al., 1981c; Wang et al., 1980; Mermelstein et al., 1981). The reduction of the nitro function was monitored spectroscopically as well as by the formation of the expected colored (blue-brown) $FeCl_3$–hydroxylamine complex.

1,8-Dinitronaphthalene (1,8-DNN), unlike other dinitronaphthalenes, is not mutagenic for *Salmonella typhimurium* (McCoy et al., 1981c; Mermelstein et al.,

TABLE 4

MUTAGENICITY OF CHEMICALLY REDUCED 1,8-DINITRONAPHTHALENE

Expt.	Procedure	Addition	Amount (μg)	Revertants per plate (TA100)
I	Plate incorporation	None	0	81
		Zn dust	114	112
		1,8-DNN	29	94
			57	118
		1,8-DNN+Zn	14	339
			29	Toxic
II	Pre-incubation	None	0	105
		Zn dust	228	112
		1,8-DNN	29	166
			57	149
		1,8-DNN+Zn	29	355
			57	Toxic
III	Pre-reacted	None	0	84
		Zn dust	114	86
		1,8-DNN	7	95
			14	77
			29	117
			114	115
		1,8-DNN+Zn	7	111
			14	117
			29	176
			57	244
			114	330

Expt. I: The reaction mixture contained 1.14, 1.14 and 0.29 mg per ml 1,8-DNN, Zn dust and NH_4Cl, respectively. Portions of this mixture were incorporated into the agar overlay.

Expt. II: The reaction mixture contained 4.57, 4.57 and 1.14 mg per ml 1,8-DNN, Zn dust and NH_4Cl, respectively. Portions of this mixture were added to 0.1 ml of bacterial culture (overnight), incubated at 37°C for 1 h, whereupon 2 ml of soft agar was added and the samples processed in the usual manner.

Expt. III. A reaction mixture consisting of 1,8-DNN (1.14 mg per ml), Zn dust (1.14 mg per ml) and NH_4Cl (0.57 mg per ml) was incubated at 37°C for 24 h and portions were removed for the determination of mutagenic activity.

1981; and Table 2). In view of the structural similarity between 1,8-DNN and 8-NQ, we sought to determine whether this lack of genetic activity was also due to an inability of the bacterial nitroreductase to reduce 1,8-DNN to hydroxylamine derivatives. As with 8-NQ, chemical reduction with zinc dust resulted in the generation of a mutagenic product (Table 4). However, unlike the putative 8-hydroxylaminoquinoline, the reduction product of 1,8-DNN could be generated in situ in the agar overlay (Table 4, Expt. I); moreover, maximum yields of the mutagenic product did not require anaerobiosis, as presumably the hydroxylamine was not greatly oxygen-sensitive. That the mutagenicity was not due to a nitro function could be demonstrated by the fact that full activity was evident in strain TA100NR, the nitroreductase-deficient strain (results not shown). The production of the hydroxylamine derivative of 1,8-DNN was monitored spectroscopically and by the formation of a colored (bluish-purple) complex with $FeCl_3$.

The present findings indicate that although bacteria are capable of reducing a variety of nitrated chemicals to mutagens, their enzymic capabilities are not unlimited as evidenced by the finding that the non-mutagenicity of carcinogen 8-NQ for Salmonella is due to an inability of the bacteria to reduce the nitro group. This blockage could be overcome by in situ chemical reduction using zinc dust, a procedure that is compatible with the bioassay. The possibility of including this non-enzymic activation as an adjunct in determing the mutagenicity of nitro-containing chemicals that are not direct-acting [e.g. 1,8-DNN which lacks activity even in the presence of S9 (unpublished results)] is obvious, especially as the inclusion of S9 preparations to effectuate reduction of the nitro function may mask the potential mutagenicity of nitro-containing chemicals (Mermelstein et al., 1981). The obvious application of this technique to other nitro compounds is underway.

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